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14. ABSTRACT

SHG intensity combined with the F/B ratio provides unique measures of the local density of ordered collagen, as well as the characteristic length scale of this ordering. In order to fully implement SHG F/B ratio measurements to understand the dynamic ordering of collagen in living tumors, we must be able to measure this property in vivo, in intact tissue, which stops us from using a forwards detector. In this annual report, we described ongoing work developing optical methods to quantify the breast tumor collagen SHG F/B scattering ratio in intact tumors in vivo, i.e. without a forwards detector. In future studies this information will be used to determine how manipulation of gene expression by tumor associated macrophages affects collagen ordering, and to determine if SHG measurement of collagen ordering could be used as a breast tumor margin detector

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Breast Tumor, Collagen, SHG, F/B ratio, In vivo microscopy

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Introduction

The measurement of the ratio of the forward-propagating to backward propagating SHG signal (the "F/B ratio") has been used to study collagen fiber ordering in various tissue samples[1-5,7]. The F/B ratio revealed the length scale of ordering in the fibers and was able to discriminate pathological tissue from healthy tissue. This implies the attractive possibility of diagnostic tools for some diseases, and possibly for breast cancer too, based on SHG F/B ratio measurements.

Previously, SHG F/B ratio measurements were made in vitro[1,4], because a second objective lens was needed to collect forward propagating SHG signal and the tissue sample had to be dissected from the animal and sectioned to allow signal to reach the second detection lens. For clinical applications, as well as basic science applications in the in vivo setting, these requirements are problematic. Last year [6], we developed a SHG confocal imaging method. By taking SHG images over the same region of interest repeatedly through a series of confocal pinholes of different sizes, we successfully measured rat tail collagen SHG F/B ratio in vivo, i.e. while the collagen fibers were still embedded in the tail. To our knowledge this variable pinhole method was the first available imaging technique that allows one to measure the SHG F/B ratio on intact tissue samples without sectioning, using just the epi-detection objective lens.

In the aforementioned variable pinhole method, five SHG images are taken over the same region of interest, which can be a time consuming process. As a consequence, the sample must be kept stationary during the whole imaging process to avoid SHG intensity variation due to the movement of the sample. During in vivo applications, or applications on extremely thick and soft tissues, this can be problematic. In the past year, we developed a new method to measure SHG F/B ratio using a pinhole mirror, which allows the measurement to be made with a single image scan and hence avoids the problem of sample motion implicit in the previous method.

Body

We are now concluding the third year of this grant, here in the Department of Biomedical Engineering at the University of Rochester Medical Center. The work we have done for both the research and training plans are listed below:

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Research Plan

<u>Hypothesis1. The ratio of forward- to backward-scattered SHG signal can be accurately imaged and quantified in breast tumor models *in vivo* and in excised biopsy sections</u>

<u>Specific Aim 1a: Determine if the method of direct collection of forward-scattered SHG signal is feasible.</u>

This specific aim had been accomplished and discussed in detail in my past publication[4], and my first year annual report.

<u>Specific Aim 1b: Determine if the collection of forward-scattered SHG signal via a confocal aperture mirror is feasible.</u>

This specific aim was accomplished and we have improved our last year's design [6] by using a confocal pinhole mirror instead of a series of pinholes of variable sizes. In the variable pinhole method, five SHG images are taken over the same region of interest, which can be a time consuming process. As a consequence, the sample must be kept stationary during the whole imaging process to avoid SHG intensity variation due to the movement of the sample. During in vivo applications, or applications on extremely thick and soft tissues, this can be problematic. The pinhole mirror method, however, allows the measurement to be made with a single image scan and hence avoids the problem of sample motion implicit in the previous method.

Also considering the availability of the sample, in the experiment, we used rat tail in stead of breast tumor tissue as sample, since it usually takes longer time for breast tumors to grow.

1. Optical setup

SHG signal was generated by a Spectra Physics MaiTai Ti:Sapphire laser providing 100fs pulses at 80 MHz and 810 nm. A quarter waveplate (Thorlabs WPMQ05M-830) was used to convert the linearly polarized excitation beam to a circularly polarized one. The beam was then directed into an Olympus Fluoview F300 scanhead connected to an Olympus BX61WI upright microscope. The focusing objective is an Olympus UMPLFL20XW water immersion lenses (20×, 0.5 N.A.). The SHG beam was separated from the excitation beam and directed out of the scanhead by a dichroic mirror (Chroma 670 DCSX) inserted into the F300, and then imaged onto the pinhole mirror with an imaging lens. SHG that passed through the pinhole mirror was filtered by a 405nm bandpass filter (Chroma HQ405/30m-2p) and detected by a photomultiplier tube (HC125-02, Hamamatsu). SHG signal that reflected off the surrounding mirror was focused through a second 405nm filter and subsequently
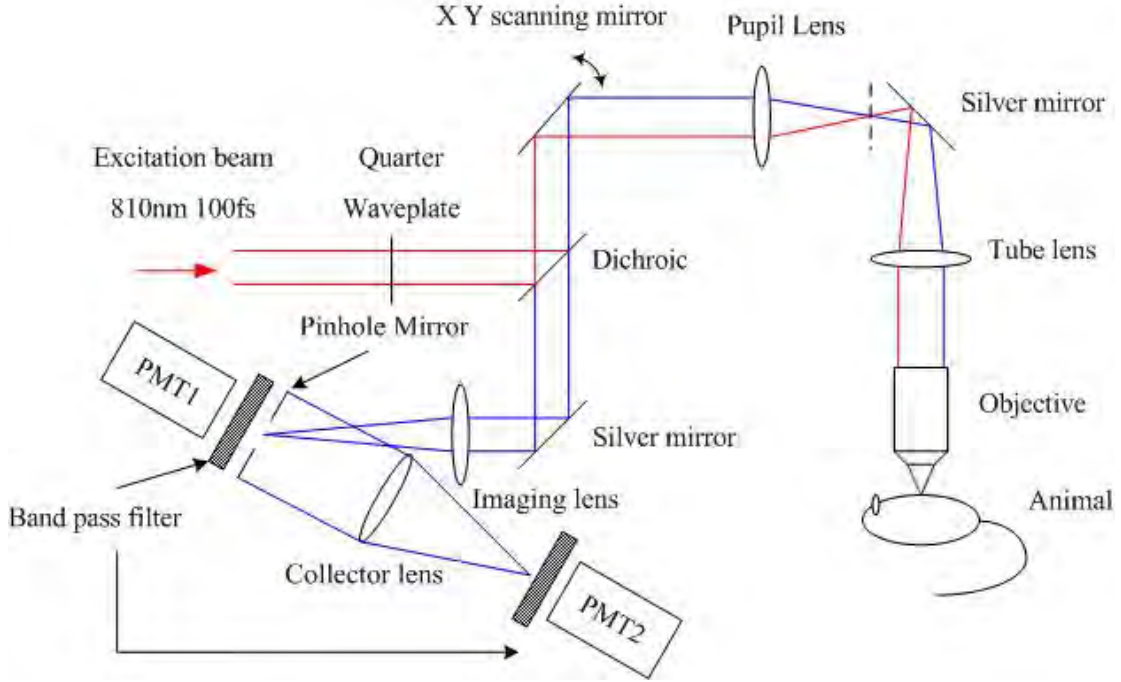
detected by a second photomultiplier tube.

Fig 1. Experimental setup for the pinhole mirror method.

The pinhole mirror is just a normal aluminum reflecting mirror, but with a pinhole of appropriate size at the center. The pinhole mirror is used here to separate the direct backward propagating SHG from the backscattered forward propagating SHG (see below). To produce this mirror, a 1.05 mm thick glass substrate was antireflection coated to minimize reflections at 405 nm (Accucoat Inc, Rochester, NY), then a 2 mm diameter droplet of oil was placed on the surface, and aluminum was deposited on its surface by vacuum evaporation using a high vacuum evaporator (Ladd Research Industries, Williston, VT). After the oil droplet was washed off, a 2 mm "pinhole" remained, with an antireflection coating on the glass within the pinhole.

2. Signal Separation

When the excitation laser beam is focused at the surface of a tissue of interest and the object plane is imaged onto a confocal plane, the spatial distribution of SHG signal on the confocal plane will consist of a sharp central peak due to the backward propagating SHG plus a diffuse signal due to the forwards propagating and subsequently backscattered SHG [6]. The image of the direct backward propagating SHG signal can be modeled as a Gaussian spot, while the forward propagating SHG that subsequently backscatters is modeled as a uniform distribution. So the total SHG signal intensity distribution on the object plane can be expressed as [6]:

$$I_{SHG}(r) = B \exp[-2(\frac{r}{\omega})^2] + FC \qquad (1)$$

Where ω is the e^{-2} Gaussian spot size of the direct backward propagating SHG, F and B are absolute intensities of forward and backward propagating SHG signals. The parameter C relates the initial forward propagating signal intensity to the average intensity of the uniform distribution of SHG light that reaches the object plane, is a function of scattering and absorption properties of the underlying tissue, and has typical values ranging between 10^{-4} and 10^{-3} [6].

From this distribution we can see the direct backward propagating SHG decays very quickly from the peak value as the distance from the laser axis increases. For example when r=3ω, the direct backward propagating SHG decays to exp(-18)=1.5x10^{-8} of its peak value. Consequently, for F/B ~1 and typical C values, the intensity of the back scattered forward propagating SHG signal is about 10^{-4}-10^{-3} of the peak intensity of the direct backward propagating SHG signal, and the direct backward propagating SHG at r≥3ω will be significantly less intense than the forward propagating SHG signal that subsequently backscatters. Therefore, if we put a pinhole mirror on the confocal plane, as long as the radius of the pinhole is ~3ω or greater, we can assume that there is no direct backward propagating SHG signal outside the pinhole. Thus, the SHG signal inside the pinhole area can be expressed by:

$$I_{pinhole} = \int_0^{2\pi} d\theta \int_0^{n\omega} \left\{ B \exp[-2(\frac{r}{\omega})^2] + FC \right\} r dr \qquad (2)$$

This signal will go through the pinhole and be detected by PMT 1. The SHG signal outside of the pinhole can be expressed by:

$$I_{Mirror} = FC(\pi R^2 - \pi n^2 \omega^2) \qquad (3)$$

Here, n is 3 or greater and represents the size of the pinhole with respect to ω, which is the SHG Gaussian spot size in the pinhole mirror plane. Note, n is not necessarily to be an integer. R is the radius of the whole mirror (if it is round), or that part of the mirror that is imaged onto the round photocathode of PMT 2. This signal will miss the pinhole and be reflected by the mirror. The collector lens will then collect the signal from the mirror and finally this signal will be detected by PMT2.

Considering the transmission and reflection coefficients of the pinhole and the mirror, we can compare the SHG intensity detected by PMT1 and PMT2:

$$\frac{P}{M} = \frac{T\%}{R\%} \frac{\frac{B}{2} \pi \omega^2 [1 - \exp(-2n^2)] + FC\pi n^2 \omega^2}{FC(\pi R^2 - \pi n^2 \omega^2)} \qquad (4)$$
$$= A[\frac{B}{F} \cdot \frac{1}{2C}(1 - \exp(-2n^2)) + n^2] \frac{\omega^2}{R^2 - n^2 \omega^2}$$

Here T% and R% represent the transmission and reflection coefficients of the pinhole and the mirror respectively, while P and M represent the detected signal in PMT 1 and 2, respectively. Since T% and R% are all constants, the ratio of these two can be represented by another constant A. We can plot this pinhole vs. mirror detected SHG ratio (P/M) as a function of the pinhole size. Typical P/M ratio vs. pinhole size plots are shown in Figure 2 below, where we assume R=12.7mm, $\omega = 350\mu m$, A~1 and C=0.0005 [6]
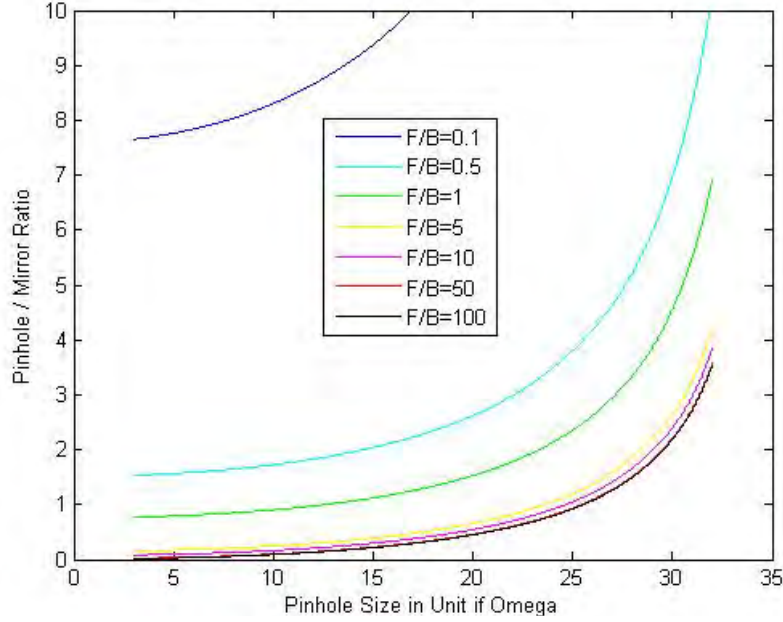

Fig 2 P/M ratio vs. pinhole size curves at different collagen SHG F/B ratios, based upon Eq. (4)

From these plots we can see that for collagen fibers with different SHG F/B ratio the shape of these P/M ratio vs. pinhole size curves are different. Since we know the actual size of the pinhole, if we can experimentally measure the ratio of the SHG intensities collected by the two different PMTs, and eliminate unknown constants by a calibration step, we would then be able to determine the real SHG F/B ratio. We can also see that when the SHG F/B ratio is larger than ~5, the P/M ratio vs. pinhole size curves are too close to each other to distinguish given typical experimental noise. This suggests that this method can determine the F/B ratio, but might only be applicable for samples with SHG F/B ratios less than ~5.

A convenient simplification arises if we consider the term in brackets in equation (4). When the SHG F/B ratio is smaller than 5 and n=3, $\frac{B}{F} \cdot \frac{1}{2C}(1 - \exp(-2n^2))$ is much larger than n^2 for typical values of C. By neglecting the n^2 term, i.e. the forward-propagating and subsequently backscattered SHG that gets through the pinhole, the P/M ratio is inversely proportional to the SHG F/B ratio:

$$\frac{P}{M} = \frac{B}{F} \cdot [\frac{A}{C} \cdot \frac{(1 - \exp(-2n^2))\omega^2}{2(R^2 - n^2\omega^2)}] \qquad (5)$$

This simplification streamlines data analysis while producing less than 10% systematic error in the worst case (i.e. when F/B is 5).

3. Pinhole and Mirror images

To evaluate our new method we applied it to the rat tail tendon, since it is a sample whose SHG properties have been well studied[1]. A whole rat tail was removed from a previously sacrificed animal. To generate a clear SHG image of the tendon, we peeled a thin layer of outer skin off the rat tail at the location of interest and exposed the tendon beneath it. We then put the rat tail on a glass slide, with the exposed collagen fiber facing up and we put another coverslip on top of the collagen fibers to assist in maintain a meniscus for our water immersion objective. The rat tail and coverslip were then fixed on the glass slide with plastic tape and the collagen fibers were imaged though the coverslip.

We prepared whole rat tail samples from 4 separate animals. On each rat tail we chose 5 image fields. And for each image field we generate two SHG images simultaneously. One SHG image was generated by PMT1, which detected SHG signal that passed through the pinhole. The other SHG image was generated by PMT2, which detected SHG signal that was reflected by the mirror. For each rat tail, we also generated one image with no sample in order to quantify the background noise. One example of these image pairs is shown below. The bright spot in the image is a fluorescent bead for calibration (see below).

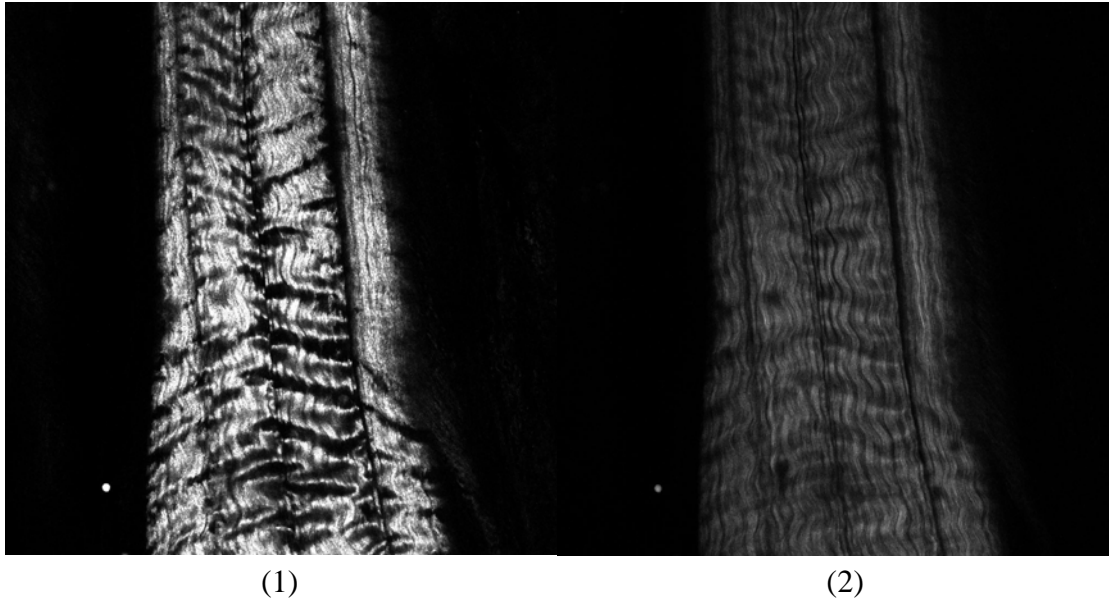

(1)   (2)

Fig 3. Pinhole(1) and Mirror(2) images of rat tail tendon collagen fibers, The bright spot is a blue fluorescent polystyrene beads, which is resting on tendon tissue below the image plane, for calibration. Images are 600 um across.

4. Calibration beads and prediction of collagen SHG F/B ratio

In order to derive the true F/B ratio from these measurements, the different optical absorption encountered by photons in the mirror and pinhole pathways as well as any difference in sensitivity of the two pathways must be accounted for. Because of these efficiencies, the detected P/M ratio deviates from the true value.

$$\left(\frac{P}{M}\right)_{\det ect} = \alpha \cdot \left(\frac{P}{M}\right)_{true} \qquad (6)$$

α here represent the efficiencies of the detection pathways and the PMTs. This means that the ratio of the detected SHG signals is represented by:

$$\left(\frac{P}{M}\right)_{\det ect} = \frac{B}{F} \cdot [\frac{\alpha A}{C} \cdot \frac{(1-\exp(-2n^2))\omega^2}{2(R^2-n^2\omega^2)}] \qquad (7)$$

Hence to determine F/B we must determine the quantities in the square brackets. While most of these (α, A, n, R, ω) are dependent upon the instrument and are not expected to vary considerably, the C factor is a function of tissue scattering and absorption underneath the image plane and may vary considerably from sample to sample.

To solve this problem, we applied a dilute solution of 10µm diameter blue fluorescent polystyrene beads (10m365415, Invitrogen) to the surface of our sample. Since the unknown constants are the same for both collagen and beads, the P/M ratio is inversely proportional to SHG F/B ratio:

$$\frac{\left(\frac{P}{M}\right)_{\det ect,collagen}}{\left(\frac{P}{M}\right)_{\det ect,beads}} = \frac{(F/B)_{beads}}{(F/B)_{collagen}} \qquad (8)$$

The P/M ratio of beads and collagen can be calculated from pinhole and mirror images, and the true F/B ratio of beads can also be conveniently measured separately, we can than calculate the true F/B ratio of collagen fibers with equation (8).

To quantify the P/M ratio from pinhole and mirror images, the background signal was first subtracted from the individual pinhole and mirror images, then the P/M ratio of all pixels was calculated. In regions of the original pinhole or mirror images where there was no significant collagen SHG or bead TPEF the P/M ratio fluctuates due to small variations in background noise around a pixel count of zero, and lacks physical

meaning. Consequently, an intensity threshold was chosen based upon the pinhole image, producing a binary image mask which set the background pixels to zero and the foreground (e.g. collagen) pixels to one. This mask was multiplied by the P/M ratio image, setting the varying background pixels to zero. Image J was then used to calculate the average pixel count of the resultant masked P/M image, and this was divided by the average pixel count of the binary mask image, producing the average P/M ratio of all pixels within fibrils or beads (i.e. all pixels above threshold in the original pinhole image). The P/M ratio was calculated for both beads and collagen separately and then the collagen SHG F/B ratio can be determined by equation (8), using the known bead F/B ratio.

As described above, we measured rat tail collagen SHG F/B ratios in 4 tails. For each tail we picked 5 ROIs and for each ROI we generated one pinhole image and one mirror image simultaneously. We then calculated P/M ratio for both the beads and the collagen as described above, and determined the average collagen SHG F/B ratio in that ROI from equation (8). The collagen SHG F/B ratio value for each of the 4 tails is then an average of the average SHG F/B ratio in 5 ROIs. The results are listed in the table 1 below:

	Animal I	**Animal II**	**Animal III**	**Animal IV**	**Average**
F/B ratio	1.66 ± 0.17	0.98 ± 0.27	1.65 ± 0.34	1.14 ± 0.43	1.36 ± 0.35

Table 1. Results of rat tail collagen SHG F/B ratio measured *in vivo* with pinhole mirror method

For each animal, means ± standard deviations are presented from 5 ROIs. The average value for the 4 animals is then presented ± the standard deviation.

To verify the validity of our new method, we need to compare the results to traditional directly measured rat tail collagen SHG F/B ratio. In the traditional direct measurement, we use both a forward and a backward detector and the sample was sectioned in ~10um thin slices. This measurement was performed previously on 5 tails, producing an average value of 1.76±0.45 (mean ± standard deviation)[6] To compare the results from the new method to that from the traditional direct measurement we performed the Kolmogorov-Smirnov test, producing a p-value of 0.2857143, indicating that these two sets of data are not significantly different from each other. This suggests that the new method produces an accurate measurement of F/B using a single epidetection objective lens, and does so from a single image scan.

Hypothesis 2. The ratio of forward- to backward-scattered SHG signal accurately quantifies collagen turnover in breast tumors models *in vivo.*

<u>Hypothesis 3</u>. The F/B ratio predicts tumor metastasis.

By reviewing the results of the past three years' research work, we realized that instead of being a metastasis detector, this technique can be applied as a breast tumor margin detector.

As long as the collagen SHG F/B ratio in breast tumor tissue is different from that in healthy tissue, this *in vivo* SHG F/B ratio measurement technique can be used to develop an optical device which can help surgeons to differentiate cancer tissue from healthy tissue during surgery. With the help of this device, the surgeons would have an improved ability to differentiate cancer tissue from healthy tissue during the operation process. The samples then sent to pathology for evaluation of margins would thereby be more likely to have positive margins, reducing the need for subsequent surgeries and hence improving quality of life.

With the traditional two lens direct method, we measured collagen SHG F/B ratio in human DCIS sample and compared the result to that in healthy tissue. As shown in figure 4, our preliminary data shows that average collagen SHG F/B ratio in human DCIS sample is statistically significantly different from that in healthy sample.
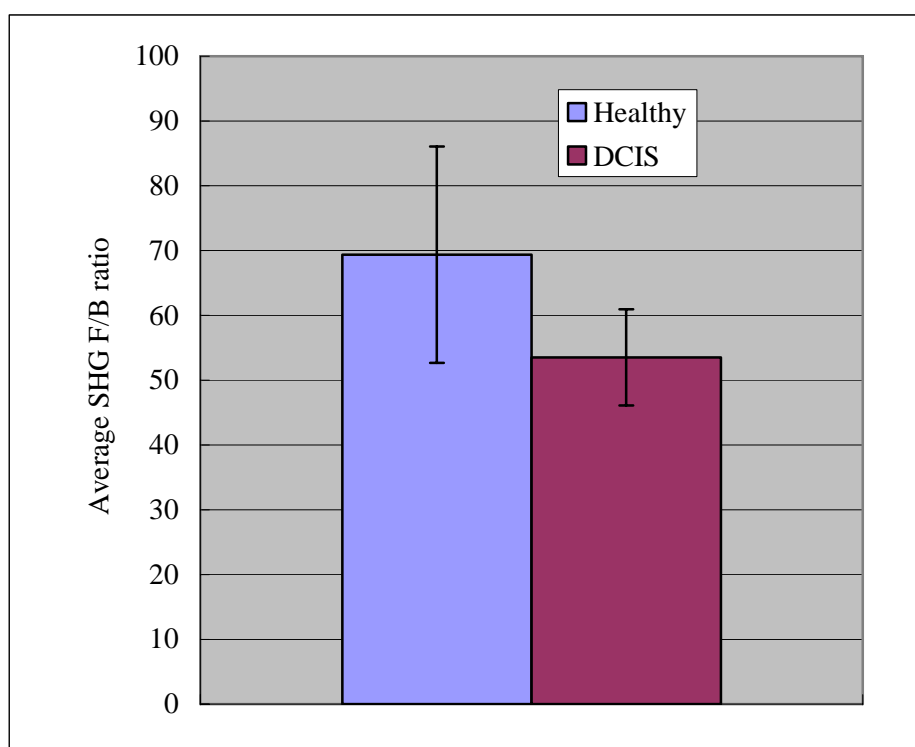

Fig 4 Average collagen SHG F/B ratio in human DCIS sample and healthy sample. Samples were paraffin embedded, sectioned, mounted and subject to SHG F/B quantification, while adjacent sections were H+E stained and classified by Dr. Ping Tang, a certified clinical pathologist. N=4 and 6 DCIS and healthy tissue, respectively, $p < 0.05$.

That means this *in vivo* SHG F/B ratio measurement technique can be used as a margin detector, and help surgeons to differentiate breast cancer tissue from healthy tissue during a breast tumor surgery

Training Plan

Task 1. Continue formal education in oncology

I have completed all proposed training courses in oncology.

Task 2. Continue formal education in optics.

I have completed all proposed training courses in optics.

Task 3. Continue informal education in oncology.

I have learned a lot about tumor biology by listening to the presentations in our group meeting every week and attending the local journal club and lectures.

Task 4. Continue informal education in optics.

I have attended OSA BIOMED and Frontier in Optics (FIO) conferences and contributed poster presentations in those meetings. I regularly attended the local journal club and lectures at the Institute of Optics. I also attended our group meeting every week.

Task 5. Laboratory training.

I have learned to prepare the dorsal skin fold chamber and grow passage and implant breast tumor cells. I am familiar with biological techniques such as northern blotting and histological stains. I am experienced in optical alignment and all other optics experiments. And I am capable to do project design, experiment design, trouble shooting and data analysis.

Key Research Accomplishments in the Past Year

1) We have built up a confocal pinhole mirror system to measure the tumor collagen SHG F/B ratio only in the backward direction.
2) We have made a pinhole mirror to separate the direct backward propagating SHG from back scattered forward propagating SHG signal.
3) We have analyzed the theoretical model to relate the measurable collagen SHG P/M ratio to the collagen SHG F/B ratio.
4) We have successfully measured rat tail collagen SHG F/B ratio in vivo with only one epi-detection objective lens and with the collagen fiber still embedded in the tail.
5) With the traditional two lens direct method, we measured collagen SHG F/B ratio in human DCIS sample and compared the result to that in healthy tissue. We found out that average collagen SHG F/B ratio in human DCIS sample is statistically significantly different from that in healthy sample.

Reportable outcomes

During the past year, a new Journal of Biomedical Optics paper was submitted
Xiaoxing Han and Edward Brown, "Epi-detected ratio of forward-propagating to back-propagating second harmonic signal with a pinhole mirror," Journal of Biomedical Optics submitted, 2011

We are invited to contribute a chapter in a new book
Han X, Perry S, Brown E Second Harmonic Imaging of Tumors. In: Campagnola P, Pavone F (eds). Second Harmonic Generation Imaging. Taylor and Francis Press. In Preparation.

We are currently applying for a new US patent:
Han X, Brown E (2010) Methods for determining the intrinsic ratio of forward-emitted to backward-emitted second harmonic generation signal with a single epidetection objective lens. Provisional Patent Application 61/330,619

Conclusion

During the past year, we have built up a confocal pinhole mirror system to measure the tumor collagen SHG F/B ratio only in the backward direction. We made a pinhole mirror to separate the direct backward propagating SHG from back scattered forward propagating SHG signal and analyzed the theoretical model to relate the measurable collagen SHG P/M ratio to the collagen SHG F/B ratio. We then successfully measured rat tail collagen SHG F/B ratio in vivo with only one epi-detection objective lens and with the collagen fiber still embedded in the tail. With the traditional two lens direct method, we measured collagen SHG F/B ratio in human DCIS sample and compared the result to that in healthy tissue. We found out that average collagen SHG F/B ratio in human DCIS sample is statistically significantly different from that in healthy sample. That means this *in vivo* SHG F/B ratio measurement technique can be used as a margin detector, and help surgeons to differentiate breast cancer tissue from healthy tissue during a breast tumor surgery

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